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14. ABSTRACT
Homologous recombination is a key cellular pathway to repair or tolerate complex DNA damage such as DNA double-stranded breaks, interstrand DNA crosslinks, or single-stranded DNA gaps. In addition, homologous recombination is required for the recovery of stalled or broken replication forks. The significance of these functions is highlighted by the use of physical (ionizing radiation) and chemical (topoisomerases inhibitors, interstrand crosslinkers) agents as principal modalities in anti-tumor therapy. The importance of the homologous recombination pathway for breast cancer is underlined by the critical function of the breast cancer tumor suppressor protein BRCA2 in RAD51 filament assembly, a central step in recombination. The objective of the research is to isolate small molecule inhibitors of homologous recombination to allow selective ablation of this pathway. Specifically, we are performing high throughput screens for two target proteins: the double-stranded DNA motor protein RAD54 and the DNA structure-selective endonuclease MUS81-EME1. We have completed a small molecule inhibitor screen for human RAD54 and are currently testing the specificity and *in vivo* effects of the lead candidates. For MUS81-EME1, we have designed and validated a fluorescence resonance energy transfer assay for the MUS81-EME1 nuclease and established the feasibility of this assay in FRET and QUENCH modes.

15. SUBJECT TERMS
Homologous recombination, small molecule inhibitor, high-throughput screen

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Table of Contents

Page

Introduction:
The project "Exploiting Synthetic Lethal Relationships: Chemical Inhibition of Recombinational Repair as a Strategy to Selectively Target Tumor Cells" aims to isolate small molecule inhibitors of homologous recombination. The specific targets are the dsDNA motor protein RAD54, a potent ATPase, and the DNA structure-selective endonuclease MUS81-EME1. The purpose of isolating inhibitors of recombinational repair is to sensitize tumor cells towards therapeutic modalities that are based on DNA damage that is normally repaired by homologous recombination, such as interstrand crosslinks, one- and two-ended double-strand breaks, and single-stranded gaps.

Body:
This report covers the second year of this project and is structured according to the original Statement of Work.

Abbreviations:

FRET:	Fluorescence Resonance Energy Transfer
HTS:	High Throughput Screen
SMDC:	Small Molecule Discovery Center
UCSF	University of California, San Francisco

UC Davis

***Task 1 (Months 1-3)*:**	*In vitro* mutagenesis.
Deliverables:	Mutant genes *RAD51-K133R, RAD54-K189R and* MUS81-D307A in *E. coli*

The *RAD51-K131R* mutant was kindly provided by Dr. K. Knight (University of Massachusetts Medical School) as a clone in pET15b, a bacterial expression vector used for RAD51 protein purification. The *RAD54-K189R* mutant (generated by Xuan Li in the laboratory) has been cloned into pFastBac1 downstream of a GST tag and the PH promoter for transposition into a bacmid in DH10 Bac *E. coli*. Sucheta Mukherjee from the Pharmacology and Toxicology Graduate Group is focusing on MUS81-EME1 and expects quick progress on making the necessary MUS81-EME1 clones and mutants soon.

***Task 2 (Months 1-36)*:**	Protein purification for assay development, HTS, secondary assays and screens
Deliverables:	Purified proteins: RAD51, RAD51-K133R, RAD54, RAD54-K189R, MUS81-EME1, MUS81-D307A-EME1

We have purified sufficient quantities of human RAD51 protein for the entire project, which is active in ATPase and *in vitro* recombination assays as previously published (*1-4*). The purification of human RAD54 from baculovirus infected insect cells was established in the laboratory, and the resulting protein is as active in ATPase and *in vitro* recombination as previously published (*5, 6*). Dr. Kirk Ehmsen, the post-doctoral fellow responsible for this project, purified 17.5 mg of human RAD54 (GST fusion) from Sf9 cells, and this was sufficient for the HTS and for all subsequent biochemical analysis anticipated for characterization of the mechanism(s) by which lead candidate compounds inhibit RAD54 ATPase activity. Rather than focus on mutant proteins for re-screening/verification stages of the HTS, we decided in consultation with Dr. Arkin to address compound selectivity for human RAD54 by testing the

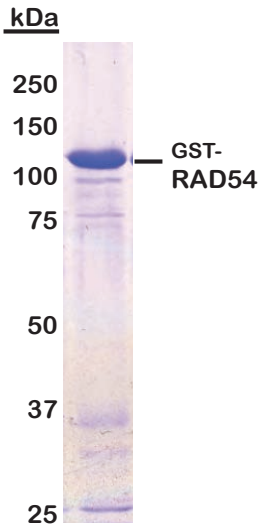

Fig. 1: Purified human GST-RAD54. Predicted M_r of GST-RAD54 is 111. 3 kDa.

lead candidate compounds identified in the HTS in malachite green and ADP-Glo ATPase assays with other well-characterized ATPases. We have therefore collected samples of a number of Swi2/Snf2 proteins (*S. cerevisiae* Rad54, Rdh54/Tid1, Chd1, & RSC; *Drosophila* Rad54; human Snf2) and several ATPases that are unrelated to the Swi2/Snf2 family (*S. cerevisiae* Srs2 & Rad51, *E. coli* RecBCD, *Drosophila* kinesin). Purification of MUS81-EME1 wild type and mutant proteins was planned for year 2 and Sucheta Mukherjee is on track to achieve this goal.

***Task 3 (Months 3-6)*:** Development of malachite green ATPase assay for RAD51/RAD54
Deliverable: HTS assay for RAD51/RAD54

Dr. Kirk Ehmsen successfully developed and optimized the malachite green ATPase assay (7) for human RAD54 in year 1 of the project plan. As indicated in last year's update, assay parameters were established in the 384-well format with two goals: (1) to maintain ATP at non-limiting concentrations up to 20 minutes reaction time, at which point the malachite green reagent is added to stop the reaction and begin color development as a function of phosphate quantity, and (2) to optimize a Z' score between 0.5 and 1.0 to validate that the detection window is sufficiently large and that variation between replicates is small. The assay was empirically optimized for ATP, RAD54, $Mg(OAc)_2$, and DNA concentration, as well as for components of the malachite green phosphate detection reagent. Figure 2 shows an example of phosphate production dependent on RAD54 concentration, for both the *S. cerevisiae* and human RAD54 proteins. Furthermore, the assay is robust in the presence of DMSO, the primary solvent in which many of the compound library molecules is delivered. Finally, the assay Z' score was optimized to 0.79, indicating that the assay was of sufficient sensitivity and reproducibility for high-throughput applications.

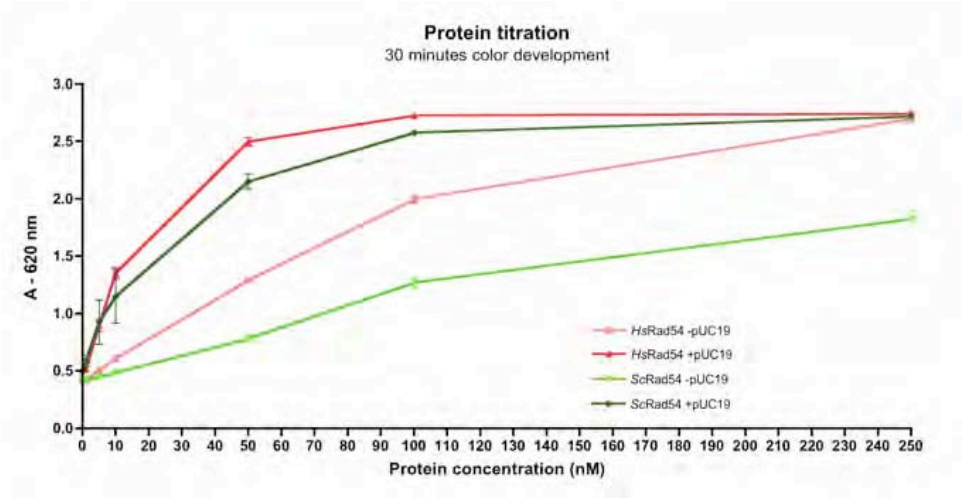

Fig. 2: Malachite green-based HTS ATPase assay. The malachite green assay reports phosphate production dependent on RAD54 concentration, defining a sensitivity window for the identification of RAD54 ATPase inhibitors.

Task 4 (Months 6-9): Pilot screen of LOPAC1280 with ATPase assay for Rad51/Rad54
Outcome: Robust assay for HTS, possible lead candidates

In consultation with Dr. Arkin in year 1, we decided to pilot the malachite green assay with compounds using the Microsource library (1,995 compounds), a collection of known bioactive compounds approved for other applications by the FDA. With post-doctoral fellow Dr. Kenny K.H. Ang of the SMDC, Dr. Kirk Ehmsen piloted the HTS with the Microsource library and confirmed that the assay was ready for a high-throughput format (Z' = 0.87 +/- 0.02). Rather than include RAD51 in the assay for RAD54 inhibitors, we focused instead on the identification of inhibitors of RAD54 ATPase on dsDNA, based on discussion with Dr. Arkin. This offered the opportunity to identify small molecules that interfere directly with RAD54 dsDNA binding or translocation, RAD54 self-interaction, or ATP binding and hydrolysis, targeting RAD54 inhibitors specifically without the further complexity associated with screening for inhibitors that interfere with the RAD54-RAD51 interaction, the RAD51-dsDNA interaction, the RAD51 self-interaction, or RAD51 ATP binding and hydrolysis.

Task 5 (Months 6-12): Development of FRET-based nuclease assay for MUS81-EME1
Deliverable: HTS assay for MUS81-EME1

Sucheta Mukherjee from the Pharmacology and Toxicology Graduate Group made considerable progress on the development of a FRET-based assay for MUS81-EME1. Of particular importance is whether the positions of the fluorescence labels in the oligonucleotide substrate interfere with endonuclease activity. Using fluorescent-labeled DNA substrates that were simultaneously labeled radioactively (^{32}P) at one 5'-end and the purified yeast Mus81-Mms4 protein, we showed in kinetic experiments that the fluorescence labels do not interfere with endonuclease activity in comparison to several control substrates (*8, 9*). By determining the kinetic parameters, K_M and k_{cat}, we show that there is no significant difference between substrates with or without fluorescent labels (data not shown). Using the yeast enzyme, we established the fluorescence-based assay in FRET and Quench mode (Fig. 3).

Tasks 6, 7 see below under UCSF

Task 8 (Months 12-15) Pilot screen of LOPAC1280 with FRET-based assay for MUS81-EME1
Outcome: Robust assay for HTS, possible lead candidates

This task is delayed by the delay in obtaining purified human MUS81-EME1 protein.

Task 9 (Months 16-36) Ongoing secondary screens to verify lead candidates and determine mechanisms

Deliverable: Possible verified lead candidates

Having identified 26 lead candidate inhibitors of RAD54, we are now prepared to further characterize them *in vitro* and to characterize them *in vivo*. We will verify the purity and identity of the 26 repurchased compounds by GC/MS, and determine the IC_{50} for RAD54 inhibition in malachite green and ADP-Glo assays (at the SMDC, UCSF). We will biochemically describe (1)

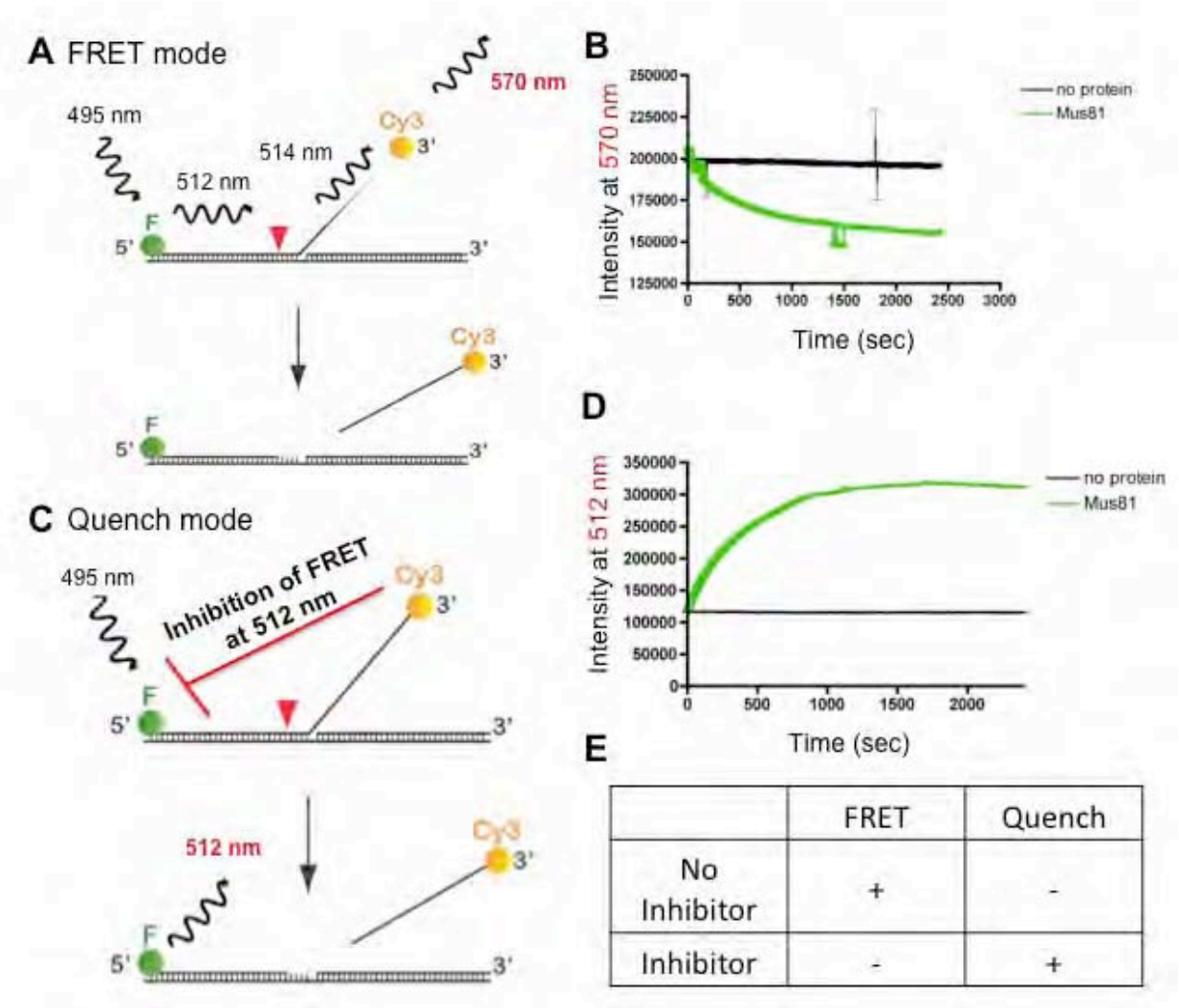

Fig. 3: Fluorescence based nuclease assay in FRET and Quench modes. (A) FRET can be detected by exciting fluorescein (F) (λ_{ex} = 495 nm) so that it emits a wavelength (λ_{em} = 512 nm) that is in the same range of excitation wavelength (λ_{ex} = 514 nm) as Cy3, so Cy3 will emit fluorescence at λ_{em} = 570 nm. 570 nm is indicated in red because it is the wavelength at which fluorescence signal is measured in the FRET mode. If *Hs*MUS81-EME1 were to incise a fluorescently labeled substrate (incision point indicated by red triangle) to create a greater distance between the fluorophores, then there will be a loss of FRET signal. (B) Preliminary data showing loss of FRET signal at 570 nm upon addition of 15 nM *Sc*Mus81-Mms4 to 25 nM fluorescent DNA substrate. (C) Cy3 is used as a quencher by inhibiting fluorescein emission. Upon *Hs*MUS81-EME1 incision, there will be increase fluorescein emission at λ_{em} = 512 nm. 512 nm is indicated in red because it is the wavelength at which fluorescence signal is measured in the Quench mode. (D) Preliminary data showing increase in fluorescein emission at 512 nm upon addition of 15 nM ScMus81-Mms4 to 25 nM fluorescent DNA substrate. (E) Table representing fluorescent signal loss (-) or signal gain (+) in FRET and Quench modes

their selectivity for human RAD54 inhibition, and (2) the mechanism by which they inhibit RAD54 ATPase *in vitro*. Selectivity will be described by testing their inhibitory action against a number of purified ATPases (Swi2/Snf2 proteins related to RAD54, as well as ATPases unrelated to RAD54; see Task 2) at 10 μM compound concentration (the screening concentration). The RAD54 inhibitory mechanism (competitive/non-competitive inhibitor; potential target site for RAD54 interaction) for each lead candidate compound will be described by conducting Michaelis-Menten kinetic assays to derive K_M and k_{cat} for ATP and dsDNA in the presence and absence of compound at the compound IC_{50} determined *in vitro*.

Task 11 (Months 12-24) Testing of verified for HTS assays
Outcome: Establishing of compounds' effect in tissue culture

In parallel to the *in vitro* characterization of lead candidate compounds, we will use HEK293 cell lines (courtesy of Dr. Jeremy Stark, City of Hope) with a genomically integrated recombination reporter system to characterize the compounds' effects *in vivo* (10). First we will test HEK293 survival in the presence of lead candidate compounds at 100 nM – 10 μM compound concentration, using a CellTiter-Glo Luminescent Cell Viability Assay (Promega) in 96-well format. Sensitization to genotoxins (MMC & cisplatin) in the presence of lead candidate compounds will be assayed by clonogenic survival assays in 6-well cluster plates at 500 ng/mL MMC or cisplatin, followed by dose response assays at a number of genotoxin concentrations (up to 1 ug/mL). Finally, for compounds that sensitize HEK293 cells to genotoxin, we will test whether the sensitization is mediated by inhibition of recombination. To do so, we will capitalize on the HEK293 integrated recombination reporter system. When these cells are transfected with a construct that constitutively expresses the I-*Sce*I endonuclease, I-*Sce*I expression induces a DNA double-stranded break (DSB) in one of two different heteroalleles of GFP. Gene conversion by intrachromosomal recombination to repair the DSB reconstitutes a functional copy of GFP. GFP expression is quantitated by FACS analysis. We expect GFP expression to be reduced in this system in the presence of compounds that interfere with recombination. To identify RAD54 inhibition as the mechanism by which recombination

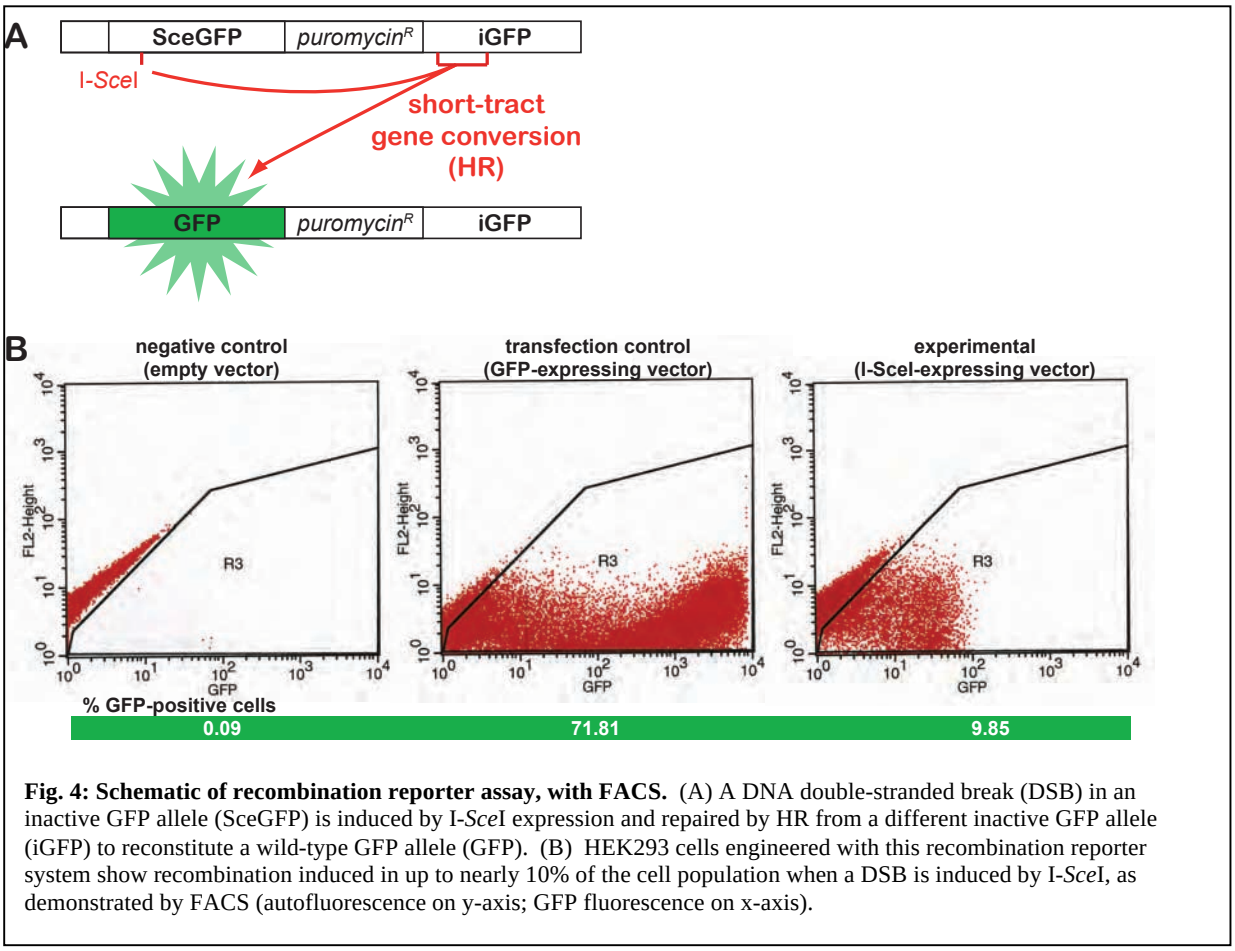

Fig. 4: Schematic of recombination reporter assay, with FACS. (A) A DNA double-stranded break (DSB) in an inactive GFP allele (SceGFP) is induced by I-*Sce*I expression and repaired by HR from a different inactive GFP allele (iGFP) to reconstitute a wild-type GFP allele (GFP). (B) HEK293 cells engineered with this recombination reporter system show recombination induced in up to nearly 10% of the cell population when a DSB is induced by I-*Sce*I, as demonstrated by FACS (autofluorescence on y-axis; GFP fluorescence on x-axis).

frequency is reduced, we will perform a variation of epistasis analysis in which recombination frequency is monitored in a combined genetic and chemical 'RAD54 knockdown' approach. Recombination frequency will be monitored in HEK293 cells in which *RAD54* expression is reduced by shRNA targeting *RAD54*, in cells in which recombination is reduced by the action of lead candidate inhibitors, and in cells treated both with shRNA and lead candidate inhibitors. We will perform similar analyses in mammalian (mouse) cell lines genetically ablated for *RAD54* and its paralog *RAD54B.* Finally, to determine to what extent lead candidate inhibitors interfere with tumor cell survival, we will test their effect on clonogenic survival of a panel of tumor cell lines, both in the absence and presence of exogenous genotoxic stress imposed by radiation or chemotherapeutics.

Based on the results of the *in vitro* and *in vivo* experiments described above, Tasks 9 & 11 have the potential to deliver lead RAD54 inhibitors that will be further developed, if necessary, along medicinal chemistry considerations (Task 12) for subsequent characterization in mammalian organismal systems (mouse models). Completion of Tasks 9, 11 & 12 will fulfill the goals of the project proposal as anticipated for human RAD54.

UCSF
Dr. Arkin
Task 6 (Months 3-36): Ongoing consultation for HTS assays

Dr. Arkin provided helpful consultation in the development of the HTS assays for RAD54 (ATPase) and MUS81-EME1 (FRET). This resulted in reassessing the original plan to conduct a pilot screen by hand, and we therefore performed the pilot assay with robotics at SMDC (UCSF) to better test the real time conditions of the HTS screen.

Dr. Arkin, Dr. Kirk Ehmsen, Dr. Kenny K.H. Ang, Dr. Adam Renslo
Task 7a (Months 9-12): HTS (8,000 compounds) with ATPase assay for RAD51/RAD54 inhibitors and troubleshooting
Deliverable: Possible lead candidates

Dr. Arkin, Dr. Kirk Ehmsen, Dr. Kenny K.H. Ang
Task 7b (Months 13-21): HTS (42,000 compounds) with ATPase assay for RAD51/RAD54
Deliverable: Possible lead candidates

These tasks were combined as recommended by Dr. Arkin.

Dr. Kirk Ehmsen & Dr. Kenny K.H. Ang performed the malachite green colorimetric screen at 10 μM compound, 2.5 nM RAD54 (monomer), and 125 nM bp dsDNA (pUC19), to identify molecules that may inhibit the ATPase activity of human RAD54 on dsDNA (overall HTS signal/background was 10.99 +/- 4.49; Z' was 0.74 =/- 0.06). We verified that under the assay conditions and time interval used, phosphate production was linear and therefore even compounds that are weakly competitive with ATP would not be lost to detection. We tested 320 of the 'hit' compounds (0.3% of the total screened, with inhibitory activity ranging from 17-99%) in an orthogonal luminescence screen, ADP-Glo (Promega). 158 compounds reduced the ATPase activity of RAD54 in both the primary screen and the orthogonal screen, and we performed dose-response curves to determine the IC_{50} (compound concentration at which 50% RAD54 activity is lost) in both malachite green and ADP-Glo formats.

To rule out DNA intercalators (which also inhibit RAD54 ATPase but by the trivial mechanism of interfering with its translocation on dsDNA, and not by direct interaction with the protein), we further characterized these 158 compounds by titrating DNA at two concentrations: 125 nM bp pUC19 (the original screening concentration) and 125,000 nM bp pUC19 (1000-fold concentration difference). We rationalized that DNA intercalators may be earmarked by a 'DNA-titratable' effect on ATPase inhibition (as observed by restoration of ATPase activity at high [DNA], and quantitated by measuring the apparent IC_{50} under the high DNA condition). We grouped these compounds into three principal classes of interest and potential mechanism of RAD54 inhibition. Class I compounds are characterized by IC_{50} values that are unchanged by DNA titration (92 compounds); Class II compounds are characterized by IC_{50} values that are lowered in the presence of high DNA concentration (28 compounds); these compounds may have particular affinity for the dsDNA-bound form of RAD54. Class III compounds are characterized by IC_{50} values that are increased in the presence of high DNA concentration (38 compounds); these compounds may (1) interact with DNA and may therefore inhibit RAD54 by a trivial mechanism (*e.g.* intercalation), or (2) these compounds may be competitive inhibitors that interact with the dsDNA-bound form of RAD54. By structural analysis, we determined that 2 of our 158 candidate compounds are, indeed, known DNA intercalators (mitoxantrone and ethidium), demonstrating that our HTS assay was susceptible to identification of DNA intercalators as false positives. To distinguish between these two possibilities for the Class III compounds, we tested all 38 Class III compounds for direct interaction with DNA. 18 DNA intercalators were identified by their ability to interfere with *E. coli* Topoisomerase I relaxation of supercoiled pUC19. We similarly tested all remaining compounds in Classes I & II for their ability to interact with DNA, and excluded from consideration those compounds that interact with DNA (27% of 158 compounds). Taking into account these data, Dr. Adam Renslo at the SMDC identified 32 compounds among our 158 candidates to repurchase as lead candidates for cell-based assays (16 compounds in Class I, 8 compounds in Class II, & 8 compounds in Class III). Of these 32 compounds, 26 are commercially available from known suppliers and have been purchased for further *in vitro* analysis and characterization in cell-based assays.

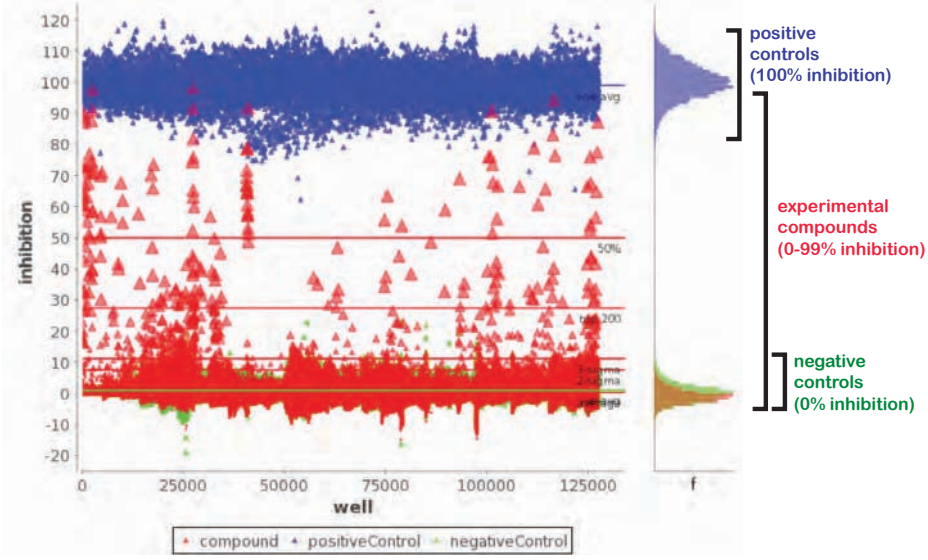

Fig. 5: Summary of HTS for human RAD54. % Inhibition is on the y-axis, and the well identity is on the x-axis (each triangle is a data point, color-coded for controls or experimental compounds as indicated to the right of the plot).

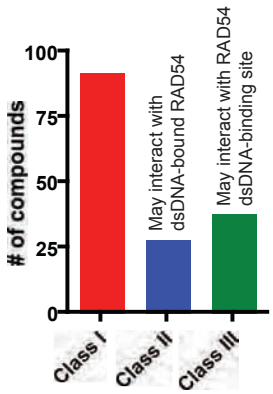

Fig. 6: Summary of classes among 158 compounds, with hypothesized mechanisms of inhibition.

Dr. Arkin and post-doctoral fellow

Task 10a (Months 18-24) HTS (8,000 compounds) with FRET-based assay for MUS81-EME1 and troubleshooting

Deliverable: Possible lead candidates

Dr. Arkin and post-doctoral fellow

Task 10b (Months 25-28) HTS (42,000 compounds) with FRET-based assay for MUS81-EME1

Deliverable: Possible lead candidates

Start is awaiting availability of purified protein.

Dr. Renslo and Dr. Mark Burlingame

Task 12 (Months 25-36) Chemistry follow-up of verified lead candidates

Once the 26 RAD54 lead candidate inhibitors are characterized *in vivo* and biochemical selectivity and mechanism of action are determined *in vitro*, Dr. Renslo and Dr. Mark Burlingame (a post-doctoral fellow at the SMDC) will address whether chemical modifications may be appropriate to better prepare the lead candidate inhibitors for assays in organismal systems.

Key Research Accomplishments:

• Completed the HTS for human RAD54 inhibitors, and identified 158 compounds that interfere with RAD54 ATPase activity in two independent formats: malachite green (colorimetric) and ADP-Glo (luminescent) assays.

• Identified (and excluded from repurchasing) DNA-interacting compounds among the 158 by DNA titration and Topoisomerase I-plasmid relaxation assays. 32 compounds were recommended for repurchasing to further characterize in cell-based survival and recombination assays. Of these, 26 are commercially available and have been repurchased.

• Piloted recombination reporter assay in HEK293 cells, which is operational for *in vivo* characterization of lead candidate compounds.

• Established the feasibility of a FRET-based assay for the human MUS81-EME1 endonuclease.

Reportable Outcomes:

None so far.

Conclusions:

We used our HTS assay for human RAD54 protein (established in year 1) to isolate small molecule inhibitors of RAD54. The HTS identified 26 lead candidate compounds from a library small molecules, and further *in vitro* analysis allowed these compounds to be grouped into three classes, two classes defined by potential mechanisms of RAD54 inhibition (interaction with the dsDNA-bound form of RAD54 or interaction with the dsDNA-binding site of RAD54). With lead candidate molecules in hand, we are now prepared to test their effect on cell survival in the presence and absence of genotoxins, and determine whether an effect on cell survival can be explained by a reduction in recombination frequency, mediated by RAD54 inhibition. We are aiming to isolate the first known small molecule inhibitors of homologous recombination, a key DNA repair pathway. Such inhibitors will be an invaluable tool for research, and moreover, have significant potential to act sensitizers in DNA damage-based tumor treatment modalities.

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